Exhibit 3

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Nonantigen Specific CD8$^+$ T Suppressor Lymphocytes Originate From CD8$^+$CD28$^-$ T Cells and Inhibit Both T-Cell Proliferation and CTL Function

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ABSTRACT: Nonantigen specific CD8$^+$ suppressor T lymphocytes (CD8$^+$ Ts) inhibit T-cell proliferation of antigen-specific T lymphocytes. The impossibility to generate *in vitro* these cells has been correlated with the appearance of relapses in patients affected by autoimmune diseases, suggesting the involvement of these cells in immune regulation. This study was aimed to identify circulating precursors and to characterize the phenotype and mechanism of action of CD8$^+$ Ts. We found that CD8$^+$ Ts can be generated *in vitro* from CD8$^+$CD28$^-$ T lymphocytes, but not from CD8$^+$CD28$^+$ T cells. A key role in their generation is played by monocytes that secrete interleukin-10 (IL-10) after granulocyte macrophage–colony-stimulating factor (GM-CSF) stimulation. Cell-to-cell direct interaction between CD8$^+$CD28$^-$ T cells and monocytes does not play a role in the generation of CD8$^+$ Ts. CD8$^+$ Ts have a CD45RA$^+$, CD27$^-$, CCR7$^-$, IL-10Rα$^+$ phenotype and a TCR Vβ chain repertoire overlapping that of autologous circulating CD8$^+$ T cells. This phenotype is typical of T lymphocytes previously expanded due to antigen stimulation. Their suppressive effect on T-cell proliferation targets both antigen presenting cells, such as dendritic cells, and antigen-specific T lymphocytes, and is mediated by IL-10. CD8$^+$ Ts suppress also the antigen-specific cytotoxic activity of CTL decreasing the expression of HLA class I molecules on target cells through IL-10 secretion. These findings can be helpful for the better understanding of immune regulatory circuits and for the definition of new pathogenic aspects in autoimmunity and tumor immunology. *Human Immunology 65, 142–156 (2004).*

KEYWORDS: tolerance; suppression; anergy; T lymphocytes; cytokines; autoimmunity

ABBREVIATIONS

CD8$^+$ Ts	CD8$^+$ suppressor T lymphocytes	TNF-α	tumor necrosis factor alpha
APC	antigen presenting cells	LPS	lipopolysaccharide
PBMC	peripheral blood mononuclear cells	PPD	purified protein derivative
DC	dendritic cells		

INTRODUCTION

Although autoreactive lymphocytes are a physiologic patrimony of our immune system [1–3], autoimmune diseases are relatively rare. The onset of autoimmune diseases is prevented by mechanisms of central and pe-

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ripheral immune tolerance among which an important role is played by regulatory cells [4–7]. These lymphocytes belong to both $CD4^+$ ($CD4^+CD25^+$) [8–10] and $CD8^+$ T-cell subpopulations [11–16] and constitute a complex system of cell subsets acting through different processes. In particular, two distinct subpopulations of $CD8^+$ T suppressor lymphocytes ($CD8^+$ Ts) have been identified. The first characterized $CD8^+$ Ts induce an antigen-specific immune suppression through cell-to-cell contact with antigen presenting cells (APC) after antigen presentation [11–13]. The second population of $CD8^+$ Ts mediates a nonantigen specific suppression of T-cell proliferation via soluble factors such as interferon-γ (IFNγ) and interleukin-6 (IL-6) [14, 15]. These lymphocytes are not cytotoxic, do not induce apoptosis, and are $CD28^-$ cells. Interestingly, the nonantigen specific $CD8^+$ Ts have been found functionally impaired in patients affected by relapsing phases of multiple sclerosis [14] and systemic lupus erythematosus [15], suggesting their possible direct involvement in the pathogenesis of autoimmune diseases. The biology of these cells presents many obscure points. In this study our findings concerning the characterization of precursors, phenotypes, and mechanisms of action of these lymphocytes are described.

MATERIALS AND METHODS

Generation of $CD8^+$ Ts

$CD8^+$ Ts were generated as described [15]. Briefly, peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on a Ficoll-Hypaque gradient for 30 minutes at 1800 rpm. PBMC were incubated in RPMI 1640 culture medium (Sigma, Milan, Italy) added with 10% fetal calf serum (Sigma), 2% glutamine (Sigma) and penicillin 100 U/ml-streptomycine 0.1 mg/ml (Sigma) in culture flasks (Corning Costar, Cambridge, MA, USA) at 37 °C overnight. After removal of nonadherent cells, monocytes were collected by gently scraping with a cell lifter (Corning Costar) and irradiated (2500 rad). $CD8^+$ T lymphocytes were purified from nonadherent cells by magnetic beads separation. In order to positively select $CD8^+$ T lymphocytes, magnetic beads coated with anti-CD8 monoclonal antibody (Dynabeads M-450 CD8; Dynal Inc., Great Neck, NY, USA) and the CD8 Detachbead (Dynal) were utilized according to the manufacturer's instructions. The positive selection procedure was repeated until the cell population was highly enriched in $CD8^+$ T cells (> 95%) as demonstrated by flow cytometric analysis. $CD8^+$ T lymphocytes (1×10^5 cells) were incubated with autologous irradiated monocytes (2.5×10^4 cells) in 96-well flat-bottomed plates (Corning Costar) in RPMI medium containing 20 U/ml IL-2 (Chiron, Emeryville, CA, USA) and 10 ng/ml GM-CSF (Roche, Milan, Italy) at 37 °C up to 7 days. At the end of the incubation, nonadherent cells were collected and the $CD8^+$ Ts population was further purified by positive selection with magnetic beads as described above. Each single $CD8^+$ Ts cell preparation was used for one single experiment.

In some experiments, allogeneic instead of autologous monocytes were used. In other experiments, generation was performed in a transwell plate thus separating autologous monocytes from $CD8^+$ T cells. Other experiments were performed in the presence of 10 μg/ml of the anti-IL-10 mAb 23738.111 (R&D System, Minneapolis, MN, USA).

In a further set of experiments, generation was performed in the absence of monocytes, incubating purified $CD8^+$ T cells with IL2 (20 U/ml) and IL-10 (40 ng/ml) (R&D System). In another set of experiments the $CD8^+CD28^+$ T cells were purified from the cell pool already enriched in $CD8^+$ cells. To this aim $CD8^+$ lymphocytes were incubated with the anti-CD28 9.3 monoclonal antibodies (mAb; 100 $\mu g/10^6$ cells) [16], and then with magnetic rat anti-mouse IgG Microbeads (Miltenyi Biotec GmbH, Bergish Gladbach, Germany) conjugated with an anti-mouse Ig monoclonal antibody. The purification was performed using the MiniMacs system (Miltenyi). The two obtained subpopulations enriched (> 95%) in $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells underwent to the procedure for the generation of $CD8^+$ Ts.

Generation of Antigen-Specific T-Cell Line

PBMC from two healthy donors were grown for 2 weeks in culture medium at a concentration of 1×10^6 cells/ml in the presence of autologous, irradiated (3000 rad) PBMC (1×10^6 cells/ml) pulsed with PPD (5 μg/ml in 200 μl of medium; Statens Seruminstitute, Copenhagen, Denmark). IL-2 (Chiron, Amsterdam, The Netherlands; 50 UI/ml) was also added to the cultures. Cells were then restimulated with autologous, irradiated and pulsed PBMC every 2 to 3 weeks, while IL2 was added to the culture every 3 to 4 days. The generation of antigen-specific T-cell lines was assessed by proliferation assays performed with resting cells. CD4 expression was demonstrated by flow cytometry using an FITC-labeled anti-CD4 mAb (Caltag Laboratories, Burlingame, CA, USA).

Generation of Dendritic Cells

PBMC were purified from heparinized blood samples of healthy donors by gradient centrifugation on Ficoll. PBMC were then plated at 2.5×10^6 cells/well in 24-well plates (Costar) in RPMI 1640 without serum at 37 °C. After 1 hour, nonadherent cells were removed by extensive washing. To check the purity of the monocyte populations, adherent cells were detached from a single well by incubation in ice-cold PBS (Sigma) supple-

TABLE 1 Percentages of $CD8^+CD28^+$ and $CD8^+CD28^-$ T lymphocytes in PBL from ten healthy patients

	Patients									
	#1	#2	#3	#4	#5	#6	#7	#8	#9	#10
$CD8^+CD28^+$	50	25	49	33	60	48	66	63	78	58
$CD8^+CD28^-$	50	75	51	67	40	52	34	37	22	42

Abbreviation: PBL = peripheral blood lymphocytes.

mented with 0.02% EDTA (Euroclone Ltd., UK), then counted and analyzed for CD14 surface expression. To obtain a population of dendritic cells (DC) capable of efficient antigen uptake, monocytes were cultured for 7 days in RPMI 1640 medium containing 5% of autologous serum, penicillin, and streptomycin (respectively, 100 U/ml and 100 μg/ml), L-glutamine (2 mM; Gibco, Grand Island, NY, USA), human recombinant granulocyte macrophage–colony-stimulating factor (GM-CSF; 25 ng/ml) and IL-4 (100 IU/ml; Euroclone, Milan, Italy). DC were further treated with tumor necrosis factor-alpha (TNF-α, 25 ng/ml; Euroclone) and lipopolysaccharide (LPS, 100 ng/ml; Sigma) in order to induce a full DC maturation [17].

PBMC and T-Cell Line Proliferation in Presence of $CD8^+$ Ts or Their Supernatant

Nonadherent PBMC were incubated with the anti-CD3 mAb UCTH-1 (5 μg/ml) at 37 °C for 2 hours, washed, counted with trypan blu dye, and seeded in a 96-well flat-bottomed plate (1×10^5 cells/well) in the presence of autologous irradiated monocytes (4×10^4 cells/well). Autologous $CD8^+$ Ts lymphocytes were added to wells at 8×10^4, 4×10^4, and 2×10^4 cells/well concentrations. Control cultures were performed in the absence of $CD8^+$ Ts lymphocytes. Some experiments were also performed in the presence of the neutralizing anti-IL-10 23738.111 mAb (R&D System) used at the concentrations of 10, 1, and 0.1 μg/ml or of an unrelated isotype-matched control mAb (R&D System). All cultures were performed in triplicate for 5 days at 37 °C. [^{3}H]-Thymidine (0.5 μCi) was added to each well 12 hours before the end of the incubation. Cells were then collected by a cell harvester and the incorporated radioactivity was detected by a β-counter (Wallac, Turku, Finland).

Experiments aimed to the evaluation of the proliferative function of antigen-specific T-cell lines in the presence of $CD8^+$ Ts were performed as follows. Resting cells of a PPD-specific $CD4^+$ T-cell line (1×10^5 cells/well) were incubated for 72 hours in 96 round bottomed microculture well plates (Corning Costar) in the presence of autologous, irradiated (3000 rad), PPD-pulsed (5 μg/ml in 200 μl of medium) DC (2×10^4 cells/well) as APC. T-cell cultures in medium alone or with unpulsed DC served as controls. The experiments were performed in the presence or in the absence of irradiated $CD8^+$ Ts cells (8×10^4, 4×10^4, and 2×10^4 cells/well). Twelve hours before harvesting, 0.5 μCi of ^{3}H-thymidine were added to each well. The radioactivity of individual wells was measured by a beta-counter. Alternatively, DC or PPD-specific T cells were either separately preincubated for 24 hours or coincubated with the supernatant of $CD8^+$ Ts cultured in fresh medium for 24 hours after generation. These experiments were also replicated in the presence of the neutralizing anti-IL-10 23738.111 mAb (R&D System) used at the concentrations of 10, 1, and 0.1 μg/ml or of an unrelated isotype-matched control mAb (R&D System). Then the cells were used for the antigen-specific proliferation assay as above specified.

Immunofluorescence Analyses

Incubations with mAb were performed at 4 °C for 30 minutes in the dark. After each round of generation of $CD8^+$ Ts the cells were first analyzed to verify the percentage of $CD8^+CD28^-$ cells. To this aim 1×10^5 cells resuspended in 100 μl of Hanks' balanced salt solution (HBSS, Sigma) were stained with an anti-CD28PE mAb (Coulter Immunotech, Miami, FL, USA) and an anti-CD8 FITC mAb (Caltag Laboratories) and then washed with PBS. Cells were then analyzed by an Epics XL flow cytometer (Beckman-Coulter Inc., Hialeah, FL, USA): the concentration of $CD8^+CD28^-$ cells was > 95% in each experiment. To further characterize the phenotype of $CD8^+$ Ts the following mAb were used: CD45RA PE, CD45Ro PE, CD27 FITC (Caltag Laboratories), CCR7 abm-2H4 (Research Diagnostic Inc., Flander, NJ, USA), anti-IL-10Rα$^-$ PE clone 37607.11 (R&D System). Isotype-matched, FITC- or PE-conjugated mAbs, specific for non relevant antigens, were used as negative controls.

The intracellular IL-10 expression by $CD8^+$ Ts was analyzed as follows. The cells (resuspended in culture medium at the concentration of 1×10^6/ml) were stimulated with PMA (50 ng/ml) and ionomicine (2 μg/ml) for 36 hours at 37 °C. Brefeldin (10 μg/ml) was added to the cells 18 hours before the end of incubation. After washings, the cells were stained with a FITC-conjugated anti-CD28 mAb (Caltag Laboratories) before fixing and

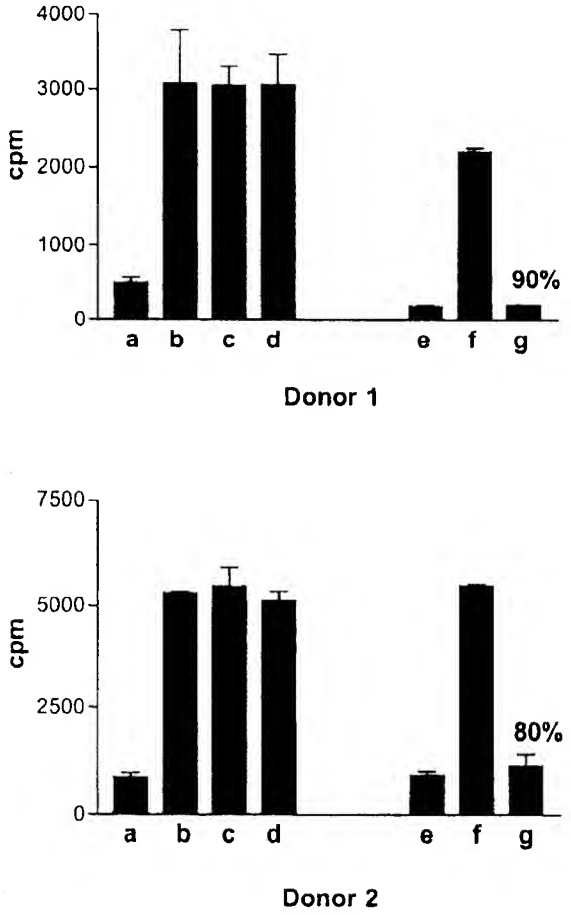


FIGURE 1 Suppressive effect of peripheral blood-derived CD8$^+$CD28$^-$ or CD8$^+$CD28$^+$ T lymphocytes and of CD8$^+$ Ts generated from CD8$^+$CD28$^-$ T lymphocytes from the peripheral blood. Two experiments are shown that are representative of six independent experiments performed with cells from different healthy donors (age 25 to 45 years old). (a and e) PBMC (1×10^5 cells/well) proliferation in the presence of autologous irradiated monocytes (4×10^4 cells/well); (b and f) as (a) and (e) plus anti-CD3 mAb UCTH-1 (5 μg/ml); (c and d) as (b) and (f) plus freshly isolated CD8$^+$CD28$^-$ (c) or CD8$^+$CD28$^+$ (d) cells (8×10^4 cells/well): lack of suppressive activity by both lymphocyte subpopulations; (g) as (b) and (f) plus CD8$^+$ Ts (8×10^4 cells/well) generated incubating CD8$^+$CD28$^-$ T cells with autologous monocytes, IL-2 and GM-CSF: a strong inhibitory effect is observed (90% and 80% for donor 1 and donor 2, respectively, as indicated on the same bar). Generation of CD8$^+$ Ts from CD8$^+$CD28$^+$ T cells was not feasible because these lymphocytes cultured with autologous monocytes, IL-2 and GM-CSF did not survive. Abbreviations: GM-CSF = granulocyte macrophage–colony-stimulating factor; IL = interleukin; Ts = suppressor T cells.

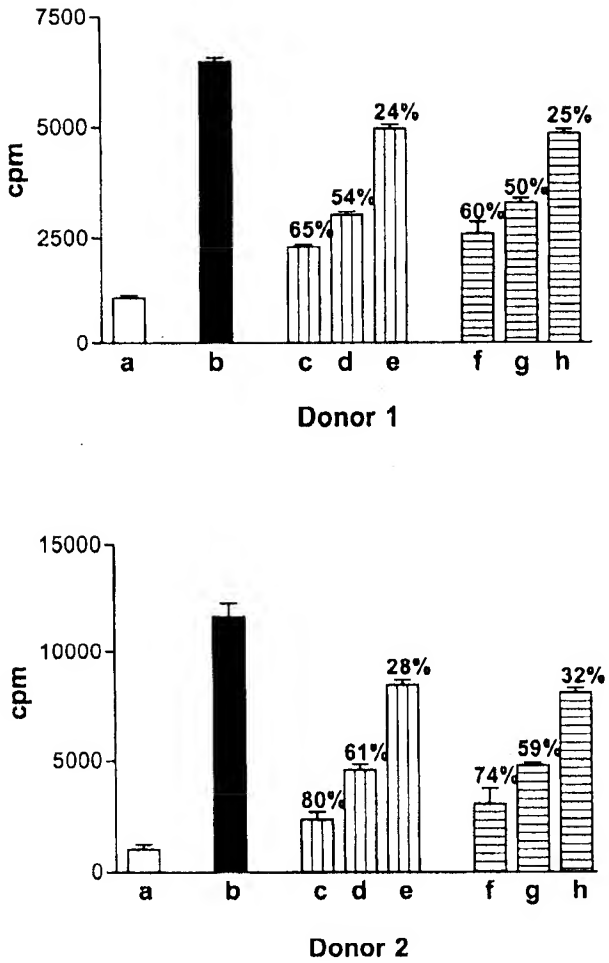


FIGURE 2 Function of CD8$^+$ Ts generated in the presence of autologous (vertical rows) or allogeneic (horizontal rows) monocytes. Two experiments are shown that are representative of six independent experiments performed with cells from different healthy donors (age 25 to 45 years old). (a) PBMC (1×10^5 cells/well) proliferation in the presence of autologous irradiated monocytes (4×10^4 cells/well); (b) as (a) plus anti-CD3 mAb UCTH-1 (5 μg/ml); (c, d, and e) as (b) plus CD8$^+$ Ts, generated incubating CD8$^+$CD28$^-$ T cells with autologous monocytes, IL-2 and GM-CSF, used at the concentrations of 8×10^4 (c), 4×10^4 (d), and 2×10^4 (e) cells/well, respectively; (f, g, and h) as (b) plus CD8$^+$ Ts, generated incubating CD8$^+$CD28$^-$ T cells with allogeneic monocytes, IL-2 and GM-CSF, used at the concentrations of 8×10^4 (f), 4×10^4 (g), and 2×10^4 (h) cells/well, respectively. The percentages of inhibition of PBMC proliferation are shown on the top of each bar. Abbreviations: GM-CSF = granulocyte macrophage–colony-stimulating factor; IL = interleukin; PBMC = peripheral blood mononuclear cells; Ts = suppressor T cells.

permeabilizing the lymphocytes with the Fix & Perm cell permeabilization kit (Caltag Laboratories) following manufacturer's instructions. The cells were then incubated with the PE-conjugated anti-IL-10 23738.111 mAb (R&D System) for 15 minues at room temperature; isotype-matched antibodies were used as controls.

The intracellular staining of macrophages was performed by incubating the cells with a TC-conjugated

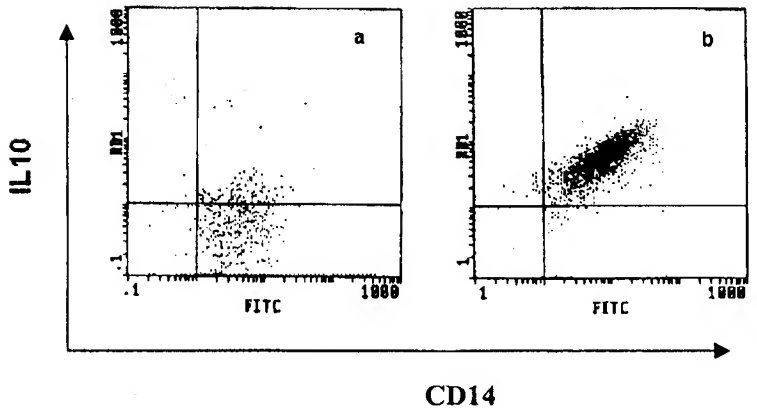


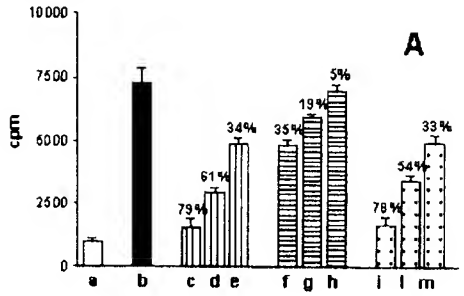
FIGURE 3 Granulocyte macrophage–colony-stimulating factor (GM-CSF) induces interleukin-10 (IL-10) production by monocytes: (a) monocytes cultured for 24 hours in culture medium; (b) monocytes cultured for 24 hours in the presence of GM-CSF.

anti-CD14 mAb (Caltag Laboratories). After washings, cells were fixed and permeabilized using the "Fix & Perm" cell permeabilization kit (Caltag Laboratories) following manufacturer's instructions, washed and incubated with the anti-IL-10 23738.111 mAb (R&D System) for 15 minutes at 4 °C. After washings, the cells were incubated with FITC-labeled goat anti-mouse immunoglobulins (Caltag Laboratories) as secondary antibody.

The phenotype of DC was analyzed by verifying the down-modulation of the CD14 antigen using the TC-conjugated anti-CD14 mAb (Caltag Laboratories,) and the expression of the CD1a antigen using the anti-CD1a mAb (Medac, Wedel, German). Analysis of cell surface markers on live cells was performed using the following mAb in immunofluorescence assays: PE-conjugated anti-CD86 (HA5.2B7, IgG2b), FITC-conjugated anti-CD80 (MAB104, IgG1), PE-conjugated anti-CD1a (BL6, IgG1), FITC-conjugated anti-CD83 (HB15A, IgG2b) (all from Immunotech, Marseille, France); anti-CD14 (M5E2, IgG2A), anti-CD16 (3G8, IgG1), and anti-CD19 (HIB19, IgG1) (all from Becton Dickinson) whereas D1.12 monoclonal antibody (IgG2a, anti-MHC class II) was kindly provided by Prof. Accolla (Insubria University of Varese). Staning with isotype-macthed antibody was performed in parallel. Direct immunofluorescence procedure was performed by diluting fluorochrome-labeled mAb with 1μg/ml human γ-globulin (human therapy grade from a commercial source), in order to block nonspecific Fc-receptor binding. Cells were then washed and the flow cytometric analysis was performed.

The analysis of the T cell receptor (TCR) Vβ chain of $CD8^+CD28^-$ T cells was performed using the IOTest

FIGURE 4 Role of IL-10 in the generation of $CD8^+$ Ts. (Panel A) Effects of an anti-IL-10 mAb on the suppressive activity of $CD8^+$ Ts; (a) PBMC (1×10^5 cells/well) proliferation in the presence of autologous irradiated monocytes (4×10^4 cells/well) (open bar); (b) as (a) plus anti-CD3 mAb UCTH-1 (5 μg/ml) (black bar); (c, d, and e) as (b) plus $CD8^+$ Ts (bars with vertical rows) used at the concentrations of 8×10^4 (c), 4×10^4 (d) and 2×10^4 (e) cells/well, respectively; (f, g, and h) as (c, d, e) plus the anti-IL-10 mAb 23738.111 (10 μg/ml) (bars with horizontal rows); (i, l, and m) as (c, d, e) plus an unrelated isotype-matched (IgG2b) control mAb (10 μg/ml) (dotted bars). The percentages of suppression of anti-CD3 mAb induced PBMC proliferation are shown on the top of each bar. (Panel B) Dose-response effect of the anti-IL-10 mAb on the suppressive action of $CD8^+$ Ts; (a) PBMC (1×10^5 cells/well) proliferation in the presence of autologous irradiated monocytes (4×10^4 cells/well) (open bar); (b) as (a) plus anti-CD3 mAb UCTH-1 (5 μg/ml) (black bar); (c) as (b) plus $CD8^+$ Ts (bar with vertical rows) used at the concentrations of 8×10^4 cells/well; (d, e, and f) as (c) plus the anti-IL-10 mAb 23738.111 (bars with horizontal rows) used at the concentrations of 10 (d), 1 (e), and 0.1 (f) μg/ml, respectively; (g, h, and i) as (c,d,e) plus an unrelated isotype-matched control mAb (dotted bars) used at the concentrations of 10 (g), 1 (h), and 0.1 (i) μg/ml, respectively. (Panel C) Comparative analysis between the suppressive functions of $CD8^+$ Ts generated by the basic procedure and that of $CD8^+$ Ts generated incubating purified $CD8^+CD28^-$ T lymphocytes with IL-2 and IL-10 in the absence of monocytes; (a) PBMC (1×10^5 cells/well) proliferation in the presence of autologous irradiated monocytes (4×10^4 cells/well) (open bar); (b) as (a) plus anti-CD3 mAb UCTH-1 (5 μg/ml) (black bar); (c, d, and e) as (b) plus $CD8^+$ Ts (bars with vertical rows) used at the concentrations of 8×10^4 (c), 4×10^4 (d) and 2×10^4 (e) cells/well, respectively; (f, g, and h) as (c, d, e) plus $CD8^+$ Ts generated incubating purified $CD8^+CD28^-$ T lymphocytes with IL-2 (20 U/ml) and IL-10 (40 ng/ml) and used at the concentrations of 8×10^4 (f), 4×10^4 (g) and 2×10^4 (e) cells/well, respectively. (Panel D) Expression of the IL-10Rα on $CD8^+$ Ts generated by incubation of purified $CD8^+CD28^-$ T cells with IL-2 and IL-10 for 7 days; (a) negative control using isotype-matched mAbs (the same control mAb was used for anti-CD28 and anti-IL-10Ra mAbs because these antibodies are both IgG1 mAbs); (b) analysis of CD8 and CD28 antigen expression: the percentage of $CD8^+CD28^-$ cells was 98%; (c) analysis of IL-10Rα expression on $CD8^+$ cells: the percentage of IL-10Rα positive cells was 98%.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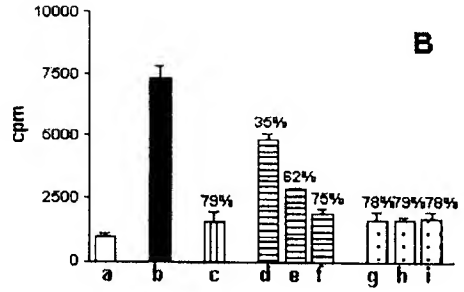
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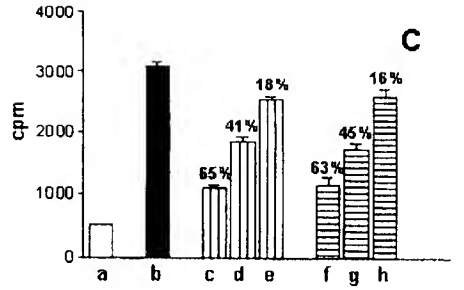
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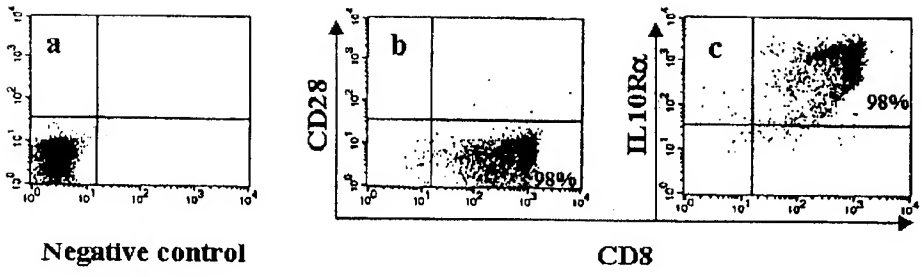
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Beta Mark, TCR Vβ Repertoire Kit (Beckman-Coulter, Marseille, France) that contains mAbs specific for the following TCR Vβ chains: Vβ5.3, Vβ7.1, Vβ3, Vβ9, Vβ17, Vβ16, Vβ18, Vβ5.1, Vβ20, Vβ13.1, Vβ13.6, Vβ8, Vβ5.2, Vβ2, Vβ12, Vβ23, Vβ1, Vβ21.3, Vβ11, Vβ22, Vβ14, Vβ13.2, Vβ4 , and Vβ7.2.

The analysis of the membrane expression of HLA-A2 molecules on T2 cells was performed using the anti-HLA-A2 BB7.2 mAb [18]. Unpulsed T2 cells and T2 cells (1×10^6 cells) pulsed with the p540 peptide of telomerase (10 μg in 150 ml of serum-free medium) were analyzed at basal condition and after 6 hour incubation with $CD8^+$ Ts supernatant (1 ml/5×10^5 T2 cells in 2 ml of culture medium) in the presence or not of the neutralizing anti-IL-10 23738.111 mAb (10 μg/ml).

Generation of p540 Specific CTL Lines

PBMC from two $HLA\text{-}A2^+$ prostate cancer patients (1.5×10^6 cells/ml) were incubated in 24 well plates in culture medium containing human AB serum and 10 μg/ml of p540 peptide. After 2 days 12 U/ml of IL-2 were added to each well. Five days later cells were collected, resuspended at 5×10^5 cells/ml, and seeded in the presence of autologous, irradiated (5000 rad) PBMC pulsed with the p540 peptide at a responder:stimulator cell ratio of 1:5. The day after 12 U/ml of IL2 and 30 U/ml of IL7 were added to the wells. After 1 to 2 weeks cells were collected, resuspended at 5×10^5 cells/ml in medium containing 30 U/ml of IL7 and plated in the presence of autologous, irradiated APC prepared as follows. Autologous, irradiated PBMC were resuspended at 4×10^6 cells/ml in medium containing both 5 μg/ml of β_2-microglobulin and 10 μg/ml of p540 peptide, and distributed in 24 well plates at 1 ml/well at 37 °C. After 2 hours non-adherent were removed and adherent cells were used as APC. The latter cycle of stimulation was repeated weekly for 5 times before analysis of CTL cytotoxicity.

Cytotoxic Assay

TAP-deficient T2 target cells (kind gift of Prof. R.S. Accolla, University of Insubria, Italy; 1×10^6 cells) were labeled resuspending them in 150 μl of $Na_2\ ^{51}CrO_4$ for 90 minutes at 37 °C. During the labelling T2 cells were also incubated (or not) with 10 μg of p540 peptide. After washings, 5×10^3/well target cells were incubated in flat-bottomed 96-well plates with CTL at a CTL: target cell ratio of 30:1. Target cells incubated with medium alone or with Triton-X100 diluted 1:100 were used to calculate spontaneous and maximum ^{51}Cr release, respectively. After 6 hours of incubation supernatants were collected and the radioactivity was detected by a gamma-counter (Wallac). The percentage of cytotoxicity was calculated as follows: percentage of lysis = sample cpm − spontaneous release cpm/maximum release cpm − spontaneous release cpm×100. In some experiments we used the anti-HLA class I W6/32 mAb (10 μg/ml) [19] or the anti-IL-10 23738.111 mAb (2 ng/ml) as blocking antibodies. In another set of experiments $CD8^+$ Ts (8×10^4 $CD8^+$ Ts/well) were coincubated with CTL and target cells during the cytotoxic assay. $CD8^+$Ts were added to the upper chamber of transwell system well plates, while CTL and T2 cells were seeded in the lower chamber, thus avoiding a direct cell-to-cell contact. In other experiments CTL or T2 cells were preincubated in transwell plates for 24 hours with $CD8^+$ Ts (8×10^4 $CD8^+$ Ts/1.5×10^6 CTL and 8×10^4 $CD8^+$ Ts/1×10^5 T2 cells, respectively) or their supernatant (1 ml/1.5×10^6 CTL in 2 ml of culture medium and 1 ml/1×10^5 T2 cells in 2 ml of culture medium, respectively).

Analysis of Apoptotic Cells

The percentage of apoptotic cells in the T2 cell population pre-incubated with $CD8^+$ Ts supernatant was assessed analyzing T2 cells by flow cytometry after incubation with biotin-conjugated annexin V (Bender Medsystem, Wien, Austria) for 30 minutes at 4 °C followed by an identical incubation with FITC-labeled streptavidin.

RESULTS

Circulating $CD8^+CD28^-$ T Lymphocytes Are Cell Precursors of $CD8^+$ Ts

$CD8^+$ Ts are phenotypically characterized to be $CD28^-$ [11, 14, 15]. It is not known whether these cells lose CD28 expression during the process of generation or if they originate from an already $CD28^-$ subpopulation. Both $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells are present at variable ratios in the circulation of healthy patients. Table 1 illustrates the percentages of $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells detected in the peripheral blood of 10 healthy donors.

We purified $CD8^+CD28^+$ and $CD8^+CD28^-$ T cell subpopulations and tested their direct suppressive activity as well as the possibility to generate $CD8^+$ Ts from each of the two subpopulations. Both freshly isolated $CD8^+CD28^+$ and $CD8^+CD28^-$ peripheral blood subpopulations were unable to mediate suppressive activity (Figure 1). Generation of $CD8^+$ Ts by 1-week incubation with autologous monocytes, IL2 and GM-CSF was only possible using the $CD8^+CD28^-$ T cells (Figure 1) since the $CD8^+CD28^+$ T cells did not survive in the same culture conditions. Thus, circulating $CD8^+CD28^-$ lymphocytes, but not $CD8^+CD28^+$ T cells, contain cells that can be committed to acquire suppressive function.

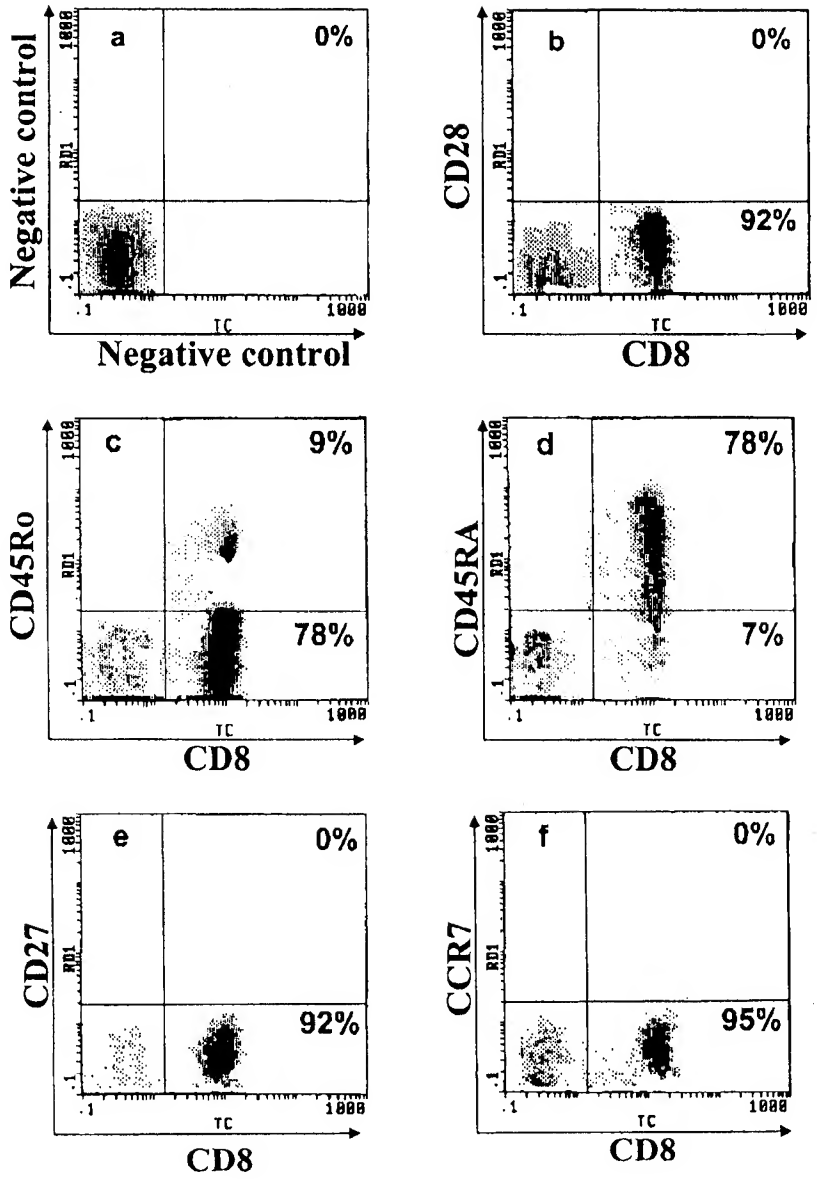

FIGURE 5 Phenotypic analysis of CD8$^+$ Ts. (Panel a) Isotype-matched, unrelated mAb were used as negative controls for the mAb specific for relevant antigens. (Panels b–f) CD8$^+$ Ts were tested by the anti-CD28, anti-CD45Ro, anti-CD45RA, anti-CD27, anti-CCR7 mAbs, respectively. The experiment is representative of three independent experiments performed with cells from different healthy donors. Abbreviations: mAb = monoclonal antibodies; Ts = suppressor T cells.

Why Monocytes Are Necessary for Generation of CD8$^+$ Ts

The fact that GM-CSF is required for the generation of CD8$^+$ Ts, although CD8$^+$ T lymphocytes do not express GM-CSF receptor, suggests that this cytokine acts on a GM-CSF receptor positive cell type, *ie*, monocytes, which affects function and/or differentiation of CD8$^+$ T cells after being stimulated by GM-CSF. Indeed, previous studies reported on the need of monocytes for the generation of suppressor CD8$^+$ T cells [20]. Accordingly, we failed in the attempt to generate CD8$^+$ Ts in the absence of monocytes since purified CD8$^+$ T lymphocytes incubated with IL-2 and GM-CSF without monocytes did not survive. It is not known the mechanism by which monocytes can commit CD8$^+$ T cells to become CD8$^+$ Ts. To clarify this point we investigated initially on the requirement for cell-to-cell direct contact. To this aim, we first compared the efficiency of generation of CD8$^+$ Ts using autologous or allogeneic monocytes. This test was conceived to explore the eventual role of HLA class I presentation. Figure 2a demonstrates that CD8$^+$ Ts, generated in the presence of either autologous or allogeneic monocytes expressed comparable suppressive functions, thus suggesting that HLA class I recognition is not involved in this process. Then, we tried to generate CD8$^+$ Ts in the presence of autologous mono-

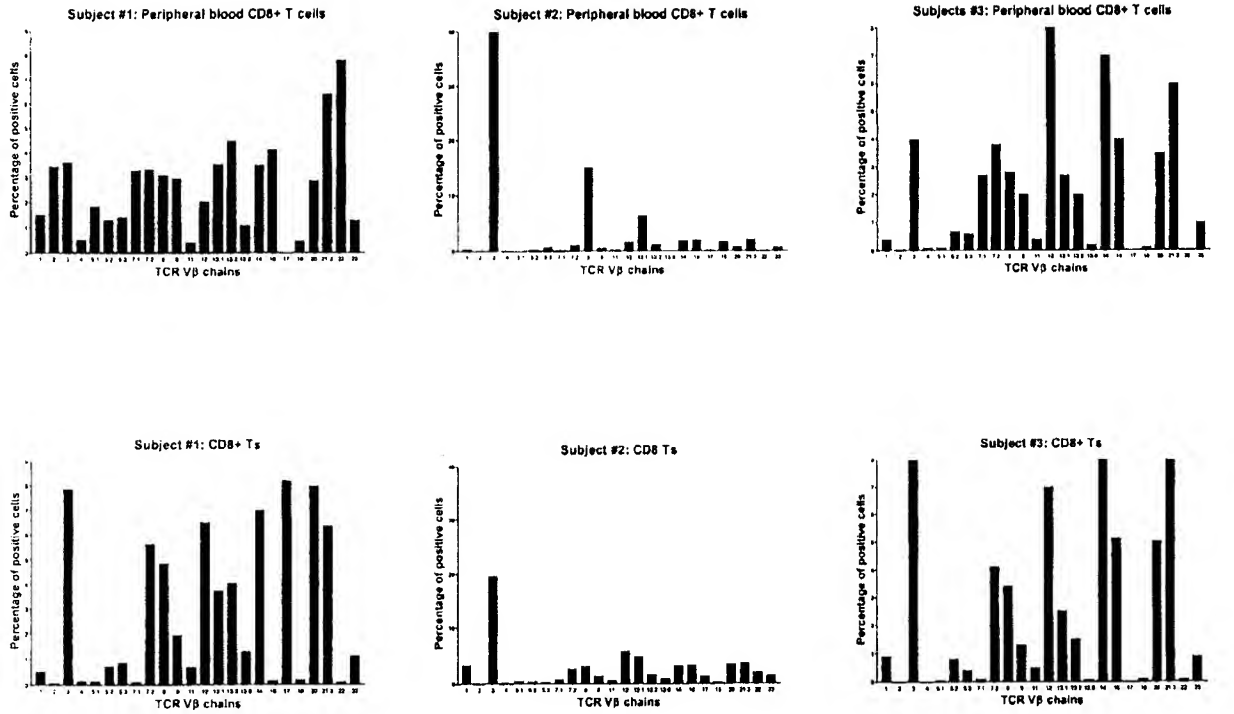

FIGURE 6 T-cell receptor Vβ chain expression by peripheral blood $CD8^+$ T cells and autologous $CD8^+$ Ts in three representative healthy patients.

cytes in a transwell system. In this condition a cell-to-cell direct interaction between $CD8^+$ T cells and monocytes is prevented by a pored membrane which still allows the passage of soluble factors. Efficient $CD8^+$ Ts were generated in this experimental setting (Figure 2b), thus underlining the possibility that a soluble factor could be at play for the generation of $CD8^+$ Ts. It is known that GM-CSF induces the production of IL-10 by monocytes [21], a finding that we could confirm (Figure 3). Consequently, we checked whether the generation of $CD8^+$ Ts could be IL-10 dependent. To this end, we used a neutralizing anti-IL-10 mAb that abrogated the generation of $CD8^+$ Ts (Figure 4a) with a dose-dependent effect (Figure 4b). In order to demonstrate that IL-10 plays a major role in the generation of $CD8^+$ Ts, experiments were performed incubating $CD8^+$ T lymphocytes with IL2 and IL-10 in the absence of monocytes. Figure 4c shows that the efficiencies of generation of $CD8^+$ Ts via stimulation of $CD8^+CD28^-$ cells by IL2 plus IL-10 or by GM-CSF activated monocytes were comparable. Accordingly, the IL-10Rα was detectable on the cell membrane of $CD8^+$ Ts (Figure 4d). All together, these data support the concept that monocytes participate to the generation of $CD8^+$ Ts by secreting IL-10 after their stimulation through GM-CSF.

Phenotype and TCR Vβ Chain Expression by $CD8^+$ Ts

In order to get insights on the stage of maturation of $CD8^+$ Ts we analyzed the expression of phenotypic

TABLE 2 Inhibition of DC antigen presentation by supernatant of $CD8^+$ Ts

			Culture conditions of DC^+ $CD4^+$			
SN of $CD8^+$ Ts	DC + PPD (5 μg/ml)	CD4 + PPD (5 μg/ml)		PPD (0.1 μg/ml)	PPD (1 μg/ml)	PPD (5 μg/ml)
No	272 ± 56[a]	1127 ± 124	3027 ± 412	9701 ± 365	12731 ± 685	18437 ± 652
Yes	25 ± 48	1082 ± 148	2003 ± 426	2936 ± 339	3127 ± 384	3210 ± 146

[a] The cpm ± standard deviation.

Abbreviations: CD4 = PPD-specific $CD4^+$ T-cell line; cpm = counts per minute; DC = dendritic cells; PPD = purified protein derivative; SN = supernatant; Ts = suppressor T lymphocytes.

TABLE 3 The inhibitory effects of CD8$^+$ Ts target both APC and antigen-specific CD4$^+$ T cells

SN of CD8$^+$ Ts	DC + PPD (5 μg/ml)	CD4 + PPD (5 μg/ml)	Culture conditions of DC$^+$ CD4$^+$			
				PPD (0.1 μg/ml)	PPD (1 μg/ml)	PPD (5 μg/ml)
No	491 ± 72[a]	270 ± 45	1425 ± 184	10571 ± 961	14724 ± 1154	16465 ± 1012
DC	—	—	1865 ± 268	2255 ± 358	3708 ± 223	5469 ± 489
CD4	—	—	1730 ± 305	2251 ± 138	2033 ± 188	2063 ± 226
DC + CD4	—	—	1365 ± 142	1482 ± 196	1398 ± 162	1374 ± 114

CD4: PPD-specific CD4+ T cell line; SN: Supernatant.

[a] The cpm ± standard deviation.

Abbreviations: APC = antigen presenting cells; CD4 = PPD-specific CD4$^+$ T-cell line; cpm = counts per minute; DC = dendritic cell; PPD = purified protein derivative; SN = supernatant; Ts = suppressor T lymphocytes.

markers related to naïve/memory/effector differentiation of T lymphocytes. Our findings demonstrate that CD8$^+$ Ts express the CD45RA but not the CD45Ro antigen. However, they cannot be considered naïve cells because they are negative for the expression of CD27 and CCR7 antigens (Figure 5).

We also analyzed the TCR Vβ chain expression of CD8$^+$ Ts and of peripheral blood CD8$^+$ T cells from which they were generated. Figure 6 reveals that the TCR Vβ chain repertoire of CD8$^+$ Ts largely overlaps that of peripheral CD8$^+$ T cells and that the TCR Vβ chain repertoires of expanded clones are different among healthy donors. All together these results suggest that CD8$^+$ Ts are already differentiated cells, hence deriving from cells that have previously undergone a process of maturation, likely after encounter with the specific antigen. Furthermore, they show that the acquisition of suppressor function by CD8$^+$ T cells is not restricted to specific TCRs.

CD8$^+$ Ts inhibit T-Lymphocyte Proliferation Targeting Both APC and Proliferating Cells

In a previous report we demonstrated that CD8$^+$ Ts inhibit T cell proliferation through the secretion of soluble factors that target in a suppressive way activated T lymphocytes [15]. However, CD8$^+$ Ts inhibitory effects could also be directed to the APC. Since DC are the most efficient APC in the body we selected this cell type to analyze the effect of CD8$^+$ Ts on antigen presentation. Initially we demonstrated that the proliferation of a PPD-specific CD4$^+$ T-cell line induced by DC antigen presentation was inhibited if performed in the presence of CD8$^+$ Ts (Table 2). Then, experiments were performed in which DC or the PPD-specific T-cell line were separately preincubated with the supernatant of CD8$^+$ Ts before testing the antigen-specific T-cell proliferation. Table 3 presents that marked inhibition of T-cell proliferation was observed in both cases at all the tested antigen concentrations. Similar findings were observed when CD8$^+$ Ts, instead of their supernatant, were preincubated with DC or the PPD-specific CD4$^+$ T-cell line before performing the antigen-dependent proliferation assay (Figure 7a). This finding supports the concept that the suppressive effect of CD8$^+$ Ts targets both antigen presenting cells and responding T lymphocytes. In the attempt to define the soluble factor responsible for the inhibitory effects exerted on APC and on responding T cells, we performed experiments using a neutralizing antibody against IL-10. Indeed, this cytokine seems directly related to the inhibition of both antigen presentation and T cell proliferation since the anti-IL-10 mAb completely counteracted the suppressive effect of CD8$^+$ Ts on both DC and the antigen-specific CD4$^+$ T cells in a dose-dependent way (Figures 7b and 7c). Accordingly with this finding, CD8$^+$ Ts were found to produce IL-10 (Figure 8).

CD8$^+$ Ts Inhibit Antigen-Specific CTL

To test whether CD8$^+$ Ts are able to inhibit antigen-specific CTL activity we generated two CTL lines recognizing the peptide p540 of telomerase (encompassing residues 540-548) in the context of HLA-A2 molecules. CTL were generated from peripheral blood of two patients affected by prostate cancer. The cytotoxicity of p540-specific CTL lines against Tap-deficient T2 cells pulsed with the p540 peptide was analyzed performing the assay in the presence or not of CD8$^+$ Ts. To avoid cell-to-cell contact CD8$^+$ Ts were separated from the rest of culture by a pored membrane using a transwell system. Figure 9, panel A shows that the cytotoxicity of the two different CTL lines was inhibited by CD8$^+$ Ts. The effect was IL-10 dependent since incubation with a neutralizing anti-IL-10 mAb counteracted the inhibition. When the experiment was replicated pre-incubating for 24 hours either CTL or T2 target cells with CD8$^+$ Ts (or with their supernatant, not shown), inhibition of cytotoxicity was observed only in the latter experimental condition (Figure 9B). This suggests that

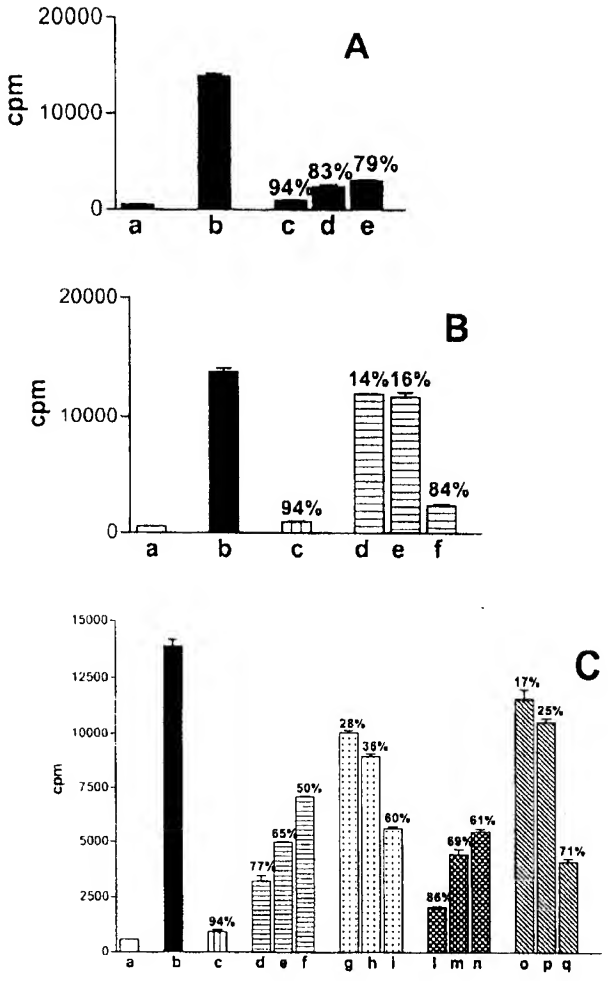

FIGURE 7 The inhibition of DC-mediated antigen presentation by $CD8^+$ Ts is IL-10-dependent. Panel A: Effects of $CD8^+$ Ts on the antigen-dependent proliferation activity of a PPD-specific $CD4^+$ T-cell line; (a) irradiated DC plus the PPD-specific $CD4^+$ T cell line; (b) irradiated, PPD-pulsed DC plus the PPD-specific $CD4^+$ T cell line; (c, d, and e) as (b) plus $CD8^+$ Ts used at the concentrations of 8×10^4 (c), 4×10^4 (d), and 2×10^4 (e) cells/well, respectively. (Panel B) The suppressive activity of $CD8^+$ Ts on antigen-dependent T-cell line proliferation is mediated by IL-10; (a) irradiated DC plus the PPD-specific $CD4^+$ T-cell line; (b) irradiated, PPD-pulsed DC plus the PPD-specific $CD4^+$ T-cell line; (c) as (b) plus $CD8^+$ Ts (8×10^4 cells/well); (d, e, and f) as (c) plus the anti-IL-10 mAb 23738.111 used at the concentrations of 10 (d), 1 (e), and 0.1 (f) μg/ml, respectively. (Panel C) The effects of IL-10 secreted by $CD8^+$ Ts target both APC and the responder T-cell line; (a) irradiated DC plus the PPD-specific $CD4^+$ T cell line; (b) irradiated, PPD-pulsed DC plus the PPD-specific $CD4^+$ T cell line; (c) as (b) plus $CD8^+$ Ts (8×10^4 cells/well); (d, e, and f) DC were preincubated for 24 hours with $CD8^+$ Ts (8×10^4 (d), 4×10^4 (e), and 2×10^4 (f) cells/well, respectively), then washed, irradiated, PPD-pulsed and used as APC for the PPD-specific $CD4^+$ T cell line; (g, h, and i) as (d, e, f) but preincubation of DC with $CD8^+$ Ts was performed in the presence of the neutralizing anti-IL-10 23738.111 mAb used at the concentrations of 10 (g), 1 (h), and 0.1 (i) μg/ml; (l, m, n): the PPD-specific $CD4^+$ T-cell line was preincubated for 24 hours with $CD8^+$ Ts (8×10^4 (l), 4×10^4 (m) and 2×10^4 (n) cells/well, respectively), then washed and incubated with irradiated, PPD-pulsed DC; (o, p, and q) as (l, m, n) but preincubation of the PPD-specific $CD4^+$ T-cell line with $CD8^+$ Ts was performed in the presence of the neutralizing anti-IL-10 23738.111 mAb used at the concentrations of 10 (o), 1 (p), and 0.1 (q) μg/ml. The experiment is representative of three independent experiments performed with cells from different healthy donors.

$CD8^+$ Ts make target cells resistant to CTL-mediated cytotoxicity. This effect was not due to lytic phenomena or apoptosis induction since viability, assessed by trypan blue staining, of T2 cells pulsed with the peptide and preincubated for 24 hours with $CD8^+$ Ts supernatant was 95% (not shown) and the percentage of apoptotic cells was ~7% (Figure 9C). The phenomenon was dependent on impaired antigen presentation as demonstrated by the fact that the expression of HLA class I molecules by T2 cells was abolished following incubation with $CD8^+$ Ts supernatant. Figure 9, panel D shows that also this effect was IL-10 dependent since it was counteracted by the anti-IL-10 mAb.

DISCUSSION

The main results of the present study are that: (a) $CD8^+$ Ts originate from circulating $CD8^+CD28^-$ T cells; (b) $CD8^+$ Ts have a phenotype and a TCR repertoire suggesting that they are preactivated and expanded clones; (c) the generation of $CD8^+$ Ts is dependent on IL-2 and IL-10; (d) $CD8^+$ Ts inhibit the APC activity of DC acting through IL-10 secretion; and (e) $CD8^+$ Ts inhibit the cytotoxic function of antigen-specific CTL by secreting IL-10.

Circulating $CD8^+$ T lymphocytes can be divided in two subpopulations, $CD8^+CD28^+$ and $CD8^+CD28^-$ T cells, whose relative percentages in the peripheral blood vary among healthy subjects ([22], and our data). To identify the precursors of $CD8^+$ Ts, we tested the possibility to generate these cells from either $CD8^+CD28^+$ or $CD8^+CD28^-$ circulating T cells. The finding that $CD8^+$ Ts were only generated from cells already lacking CD28 expression suggests that the circulating $CD8^-CD28^-$ T cell subpopulation contains the precursors for $CD8^+$ Ts. $CD8^+CD28^-$ T lymphocytes have been reported to derive from a $CD8^+CD28^+$ T-cell subpopulation that downmodulates the CD28 antigen after prolonged stimulation in long-term *in vitro* culture [23, 24]. Interestingly, it has been reported that $CD8^+$

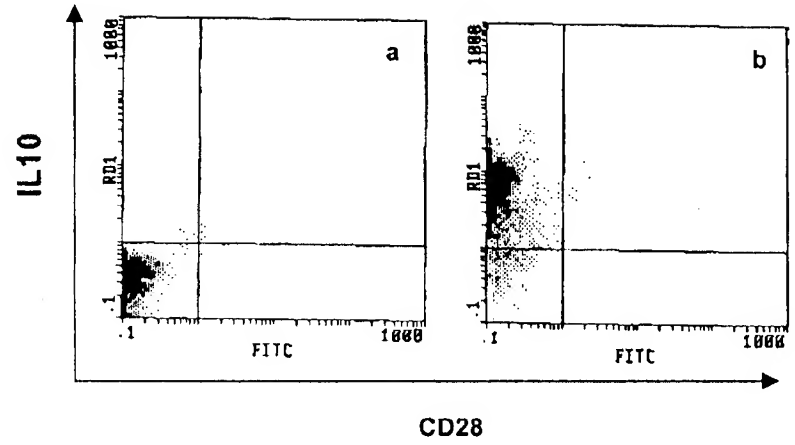

FIGURE 8 CD8$^+$ Ts cells express IL-10. CD8$^+$ Ts were stimulated with PMA and ionomicine for 36 hours. Brefeldin was added during the last 18 hours. Cells were then fixed and permeabilized before analysis by flow cytometry. Cells were stained with a PE-conjugated anti-IL-10 and a FITC-conjugated anti-CD28 mAbs (b) or with isotype-matched control mAbs (a).

T cells committed to effector function by antigen stimulation are characterized by the expression of the CD45RA antigen associated with the lack of expression of the CD27 antigen [24, 25]; moreover, CD8$^+$CD27- T cells are also CD28$^-$ and lack CCR7 expression [25]. We found that CD8$^+$ Ts are CD27-CCR7- cells, a finding supporting the idea that these cells derive from precursors repeatedly stimulated by the antigen. Accordingly, the study of the TCR Vβ chain expression showed that expanded CD8$^+$ Ts clones correspond almost completely to CD8$^+$ clones already expanded in the peripheral blood without restriction to specific TCR Vβ chains. This is conceivable since we observed that CD8$^+$ Ts generation does not require antigen presentation. However, CD8$^+$CD27$^-$CCR7$^-$ T cells described until now are cytotoxic cells, while CD8$^+$ Ts are not able to mediate cytotoxic activity [15]. Thus, our data provide support for the existence of a so far unrecognized subpopulation of CD8$^+$CD28$^-$CD27$^-$CCR7$^-$ T cells possessing suppressor function. An appealing interpretation of our findings could be that CD8$^+$ Ts represent a subpopulation of "effector" CD8$^+$ T cells specifically committed to mediate suppressor function in the presence of the adequate cytokine milieu, even in the absence of specific antigen recognition. Since the presence of CD8$^+$ Ts could not be demonstrated in the peripheral blood of healthy subjects, at variance with their precursors, the commitment to CD8$^+$ Ts might occur in the tissues at the sites of inflammatory reactions. It will be of interest in the next future to investigate on this hypothesis by trying to purify CD8$^+$ Ts from bioptic specimens of inflamed and/or neoplastic tissues.

IL-10 is a cytokine expressing pleiotropic functions [26]. Concerning the anti-inflammatory and immunoregulatory actions of this cytokine, it is relevant its involvement in the generation of regulatory CD4$^+$ [27] and CD8$^+$ [16] T lymphocytes. The demonstration that generation of CD8$^+$ Ts is also dependent on IL-10 by paracrine-autocrine mechanisms (a datum supported by the expression of the IL-10Rα by CD8$^+$ Ts) further underlines the central role played by this cytokine in the regulatory/suppressive mechanisms responsible for peripheral immune tolerance. The finding that CD8$^+$ Ts can be generated incubating purified CD8$^+$ T lymphocytes with IL2 and IL-10 is also relevant because it could open new immunotherapeutic perspectives for the treatment of autoimmune diseases. In fact, the identification of the cytokines directly responsible for the commitment of CD8$^+$ T cells in CD8$^+$ Ts can facilitate the *in vitro* generation and expansion of this cell subpopulation. Indeed, in preliminary experiments we succeeded in expanding and keeping in culture for more than 1 month CD8$^+$ Ts cells without losing their suppressive function. Since it has been shown an impairment of CD8$^+$ Ts function in patients affected by relapses of multiple sclerosis and systemic lupus erythematosus, a future attempt for the treatment of systemic autoimmune diseases might consist in the expansion of autologous CD8$^+$ Ts during quiescent phases of autoimmune diseases and their re-infusion during the relapses. In this view, CD8$^+$ Ts offer advantages with respect to the other regulatory/suppressor cell subpopulations described until now. In fact, data presented here highlight that their generation and function do not require specific antigen recognition. Hence, these cells could be utilized to restore the immunologic homeostasis in the course of autoimmune diseases independently from the autoantigen involved in the autoimmune response. Works are in progress in our laboratory to evaluate the efficacy of the immunotherapeutic approach based on the infusion of CD8$^+$ Ts in experimental models of autoimmune diseases.

The activity of CD8$^+$ Ts is more complex than previously imagined since it targets not only T lymphocytes stimulated by the specific antigen but also the antigen presenting cells. Indeed, DC preincubated with the supernatant of CD8$^+$ Ts failed to efficiently present the

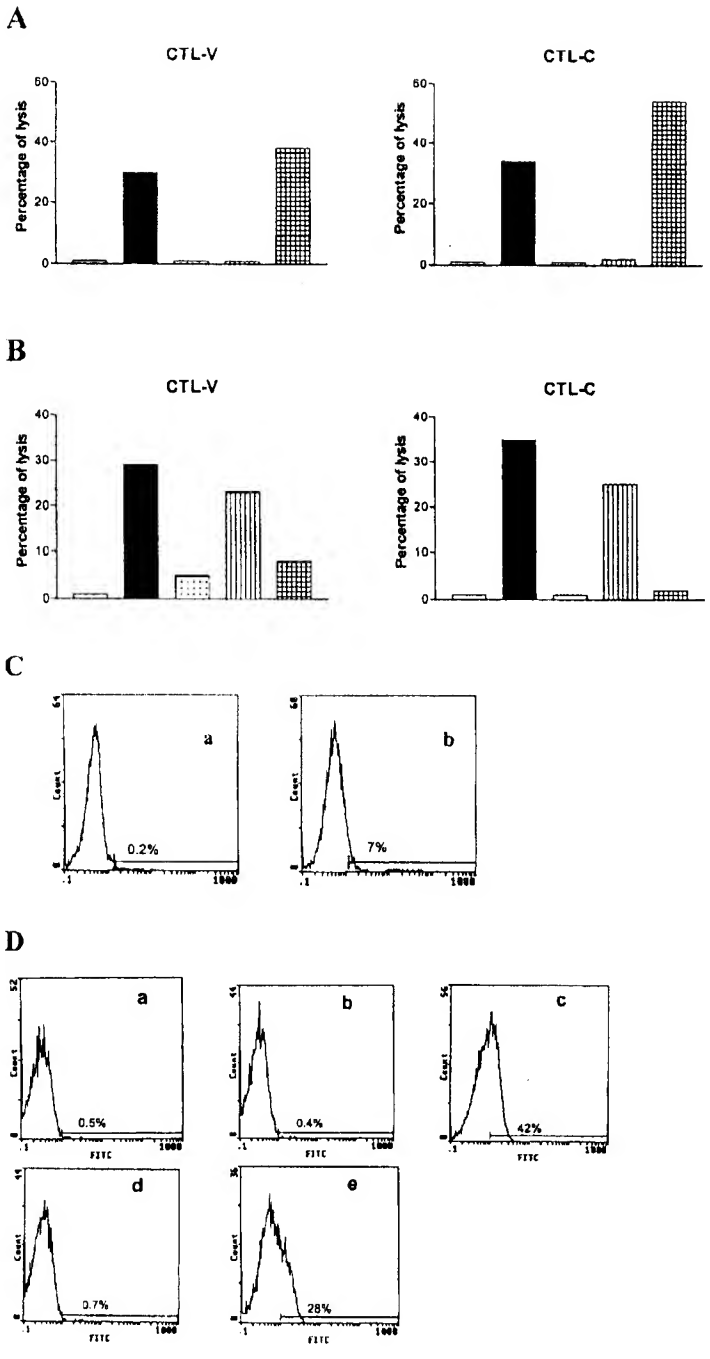

FIGURE 9 $CD8^+$ Ts inhibit the cytotoxic function of antigen-specific CTL downmodulating HLA class I membrane expression on target cells. CTL-V. and CTL-C, CTL lines specific for the p540 peptide of telomerase, were generated from two $HLA\text{-}A2^+$ prostate cancer patients. The cytotoxicity of these CTL lines was tested against T2 cells pulsed with the p540 peptide. The CTL:target cell ratio was 30:1. (Panel A) the cytotoxic activity of CTL was tested against T2 cells (open bars), T2 cells pulsed with the p540 peptide (black bars), T2 cells pulsed with the p540 peptide in the presence of the anti-HLA class I W6/32 mAb (dotted bars), T2 cells pulsed with the p540 peptide in the presence of $CD8^+$ Ts (bars with vertical rows), and T2 cells pulsed with the p540 peptide in the presence of both $CD8^+$ Ts and the neutralizing anti-IL-10 23738.111 mAb (squared bars). (Panel B) The experiments were performed incubating CTL with T2 cells (open bars), with T2 cells pulsed with the p540 peptide (black bars), with T2 cells pulsed with the p540 peptide in the presence of $CD8^+$ Ts (dotted bars), preincubating for 24 hours CTL with $CD8^+$ Ts before incubating them with T2 cells pulsed with the p540 peptide (bars with vertical rows), and preincubating T2 cells for 24 hours with $CD8^+$ Ts before incubating them with CTL (squared bars). (Panel C) Analysis of apoptosis induction in T2 cells after 24-hour incubation with $CD8^+$ Ts supernatant; (a) negative control; (b) annexin-binding T2 cells. (Panel D) Immunofluorescence analysis by flow cytometry of HLA-A2 membrane expression on T2 cells using the BB7.2 mAb; (a) FITC-labeled secondary antibody alone; (b) non-pulsed T2 cells; (c) analysis performed after 6 hours from pulsing of T2 cells with the p540 peptide; (d) p540-peptide pulsed T2 cells incubated for 6 hours with the supernatant of $CD8^+$ Ts; (e) p540-peptide pulsed T2 cells incubated for 6 hours with the supernatant of $CD8^+$ Ts in the presence of the neutralizing anti-IL-10 23738.111 mAb.

antigen to antigen-specific T lymphocytes, an effect dependent on IL-10. Thus, the inhibitory action of $CD8^+$ Ts is bi-directional since it is addressed to both APC and responding cells. Interestingly, IL-10-mediated inhibitory action on DC does not seem due to modulation of membrane antigen expression, as for other similar cell systems [13]. In fact, no variations of either percentage of positive cells or membrane density of molecules involved in antigen presentation, such as HLA-class I and II molecules, CD40, B7.1, B7.2, ICAM-1, ILT3, ILT4, were observed on DC after exposure to $CD8^+$ Ts for 24 hours (not shown). Alternative mechanisms of IL-10-mediated DC inhibition, such as the proposed increased binding of the invariant chain to HLA class II molecules

[28] or others still unknown, deserve to be investigated in the next future. Concerning the IL-10-dependent inhibition of antigen-specific $CD4^+$ T cell proliferation, it is likely related to the known capacity of this cytokine to induce anergy on activated T cells [29]. Since the activity of $CD8^+$ Ts was previously found to be in part dependent on the secretion of IL-6 and IFN-γ [15], we hypothesize that these cells mediate overwhelming suppressive signals through different cytokine-dependent metabolic pathways on different cell types (*ie*, T helper lymphocytes, dendritic cells, CTL).

The different subpopulations of $CD8^+$ T suppressor/regulatory cells as far recognized have been characterized for their capacity to inhibit T-cell proliferation. No data have been yet reported on the activity of these cells on cytotoxic functions. Here we show the unprecedented finding that nonantigen specific $CD8^+$ Ts efficiently inhibit cytotoxicity of antigen-specific CTL. This phenomenon was not due to lytic or pro-apoptotic mechanisms since viability of T2 cells exposed to the supernatant from $CD8^-$ Ts was unaffected and no relevant apoptotic events were observed. Furthermore, no expression of molecules related to cytotoxic and proapoptotic functions, such as granzymes, perforins, and FasL, was observed in $CD8^+$ Ts (not shown). We could define that the $CD8^+$ Ts-mediated inhibition of CTL cytotoxicity is an IL-10-dependent phenomenon and that it occurs through downmodulation of the HLA class I molecules at the surface of target cells. This result is in agreement with what already reported by others concerning the capacity of IL-10 to decrease the membrane density of HLA class I molecules making target cells less susceptible to CTL cytotoxicity [30].

Altogether the findings here reported suggest that the role of $CD8^+$ Ts in controlling immunologic responses is relevant since they suppress both T-cell proliferation and cytotoxicity. In particular, the inhibition of T-cell proliferation is guaranteed by a double, additive mechanism, thus suggesting the importance of the $CD8^+$ Ts-mediated control on peripheral immune responses. Interestingly, the impossibility to generate $CD8^+$ Ts has been correlated with the occurrence of relapses in autoimmune diseases such as multiple sclerosis and systemic lupus erythematosus [14, 15]. These observations support the view that $CD8^+$ Ts are involved in the maintenance of immune homeostasis and, consequently, in the development of autoimmune reactions when functionally impaired. This is further supported by the discovery that $CD8^+$ Ts are also able to suppress CTL function. This finding prompts studies on $CD8^+$ Ts activity in autoimmune diseases in which CTL play a pathogenic role. Furthermore, future studies must define the presence of $CD8^+$ Ts in the inflammatory infiltrate in the course of chronic inflammatory diseases and cancer. Preliminary experiments in our laboratory demonstrate the presence of functional $CD8^+$ Ts in metastatic lymph nodes from cancer patients. The significance of this finding could be related to the establishment of immunologic tolerance toward the tumor.

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